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Tile of the Invention: MULTICELLULAR BACTERIA DERIVED FROM NORMAL/NONDISEASED AND DISEASED MAMMALIAN TISSUES		

CERTIFICATE OF FILING BY "EXPRESS MAIL"

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I hereby certify that the attached Provisional Application for United States Patent, entitled MULTICELLULAR BACTERIA DERIVED FROM NORMAL/NONDISEASED AND DISEASED MAMMALIAN TISSUES, with accompanying Application Data Sheet and check for $80.00 covering the small entity filing fee, is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service in an envelope addressed to the Assistant Commissioner for Patents, Washington, DC, 20231, on March 17, 2004.

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Title of the Invention: MULTICELLULAR BACTERIA DERIVED FROM NORMAL/NONDISEASED AND DISEASED MAMMALIAN TISSUES

Inventor: Douglas H. Robinson

Background of the Invention

Multicellularity represents one of the major transitions in the evolution of biologic complexity[1]. Bacterial species such as *Myxococcus xanthus* exhibit characteristics of multicellular organisms including cooperative behavior among individuals and coordinated cell-cell attachment[2]. Here it is shown that pleomorphic bacteria derived from a mammalian tumor self-organize *in vitro* into mammalian tissue-like morphogenetic patterns consisting of tissue-like sheets and capillary-like networks. This bacterial multicellularity is unlike any previously reported.

Summary of the Invention

In one embodiment, the present invention relates to bacteria derived from mammalian tissues, which exhibit tissue-like multicellular self-organization when cultured *in vitro*.

In one preferred embodiment, the bacteria exhibit multicellular self-organization into tissue-like sheets when cultured *in vitro*.

In another preferred embodiment, the bacteria exhibit multicellular self-organization into capillary-like networks when cultured *in vitro*.

The multicellular bacteria of the present invention may be used to produce *in vitro* autologous or allogeneic therapeutic or prophylactic vaccines for treatment of cancer, AIDS, and other mammalian diseases.

The multicellular bacteria of the present invention isolated from apparently healthy individuals may also be used as an *in vitro* marker for impending disease.

The multicellular bacteria of the present invention may be used as an *in vitro* model to test new or existing drugs and biomolecules that may disrupt/kill multicellular bacteria integral to the pathogeneses of mammalian cancer and other mammalian diseases.

The multicellular bacteria of the present invention may be used *in vitro* as a model to test new or existing radiofrequency therapeutic modalities that may kill/disrupt multicellular bacteria integral to the pathogeneses of mammalian cancer and other mammalian diseases.

The multicellular bacteria of the present invention may be used *in vitro* as a model to test new or existing nutritional supplements that may kill/disrupt multicellular bacteria integral to the pathogeneses of mammalian cancer and other mammalian diseases.

The multicellular bacteria of the present invention may be used as 'guidewires' *in vivo* for vacularization/revascularization of diseased hypoxic mammalian tissues.

The multicellular bacteria of the present invention may be used to express novel genes/proteins, *i.e.*, chimerical (eukaryotic/prokaryotic) proteins or proteins with little homology to known proteins to be used to treat mammalian diseases.

The multicellular bacteria of the present invention may be used to express eukaryotic, prokaryotic, chimerical, and novel genes/proteins useful in the treatment of mammalian diseases.

The multicellular bacteria of the present invention may be used to stimulate angiogenesis (new blood vessel formation) *in vivo* in mammalian diseases that involve deficient blood flow to diseased tissues (cardiac, brain, muscle, etc. diseases).

The multicellular bacteria of the present invention may be used to derive mammalian angiogenesis-related genes/proteins that can be used to treat mammalian diseases that require new blood vessel formation (angiogenesis).

The multicellular bacteria of the present invention may be used as a cloning vector to express genes/proteins of interest.

The multicellular bacteria of the present invention may be used to produce *in vitro* novel antibiotics to treat mammalian diseases, including, but not limited to, cancers and infectious diseases.

The multicellular bacteria of the present invention, either viable or nonviable, may be used to promote angiogenesis and wound repair *in vivo* by placing them directly into mammalian wounds.

The multicellular bacteria of the present invention may be used to produce *in vitro* cellular components or proteins that can be used to serve as scaffolding for the deposition

of inorganic materials (gold nanoparticles and cadmium selenide-zinc suphide) into a regular array of nanoparticles for the manufacture of computer chips.

The multicellular bacteria of the present invention may be used to produce elastic proteins (bioelastomers, elastomeric proteins, or biopolymers) to treat mammalian diseases such as ligament injuries/defects, burns (a matrix for skin grafting or to promote host skin growth/repair), heart valve injuries/defects, and other disorders of connective tissue, etc.

The multicellular bacteria of the present invention may be used to produce elastic proteins (bioelastomers , elastomeric proteins, or biopolymers) and other proteins to be used in both medical and nonmedical biological materials science applications such as the production of cell-culture scaffold material for growing cells for arterial grafts, skin grafts, heart valves, etc.; synthetic ligaments; synthetic suture materials; biosensors; materials for drug delivery; coatings for catheters, leads and drainage tubes; protective body armor; acoustical absorbers; durable elastic thermoplastics, etc.

The multicellular bacteria of the present invention may be used to produce elastic proteins (bioelastomers, elastomeric proteins, or biopolymers) and other proteins *in vivo* in mammalian tissues by "infecting" such mammalian tissues with multicellular bacteria that infiltrate said mammalian tissues and produce the elastic proteins directly in the tissues.

The multicellular bacteria of the present invention may be used to identify eukaryotic genes and proteins involved in mammalian morphogenesis that can be used to treat and/or develop new treatments for various mammalian diseases such as congenital heart disease and cardiomyopathies, blood vessel abnormalities and atherosclerosis, pulmonary alveolar abnormalities, etc.

Multicellular bacteria of the present invention derived from tissues of patients with HIV infection and/or AIDS may be used to produce vaccines against HIV infection and/or AIDS.

Multicellular bacteria of the present invention derived from normal or aging mammalian tissues may be used to produce vaccines against cellular and tissue degradation associated with the aging process. It is known in the art that chronic bacterial infection "ages" host tissues. If the natural mammalian aging process involves

infection with multicellular bacteria such as those observed in connection with the present invention, then one aspect of the aging process may be a chronic inflammatory state produced by an immune system that is attempting to rid the body of infection by multicellular bacteria; this chronic inflammatory state damages cells and tissues. Inhibition or eradication of the causative multicellular bacteria would provide a protective affect against this aging-related cellular and tissue damage.

Multicellular bacteria of the present invention derived from diseased/abnormal mammalian tissues may be used to develop antibiotics and vaccines to eradicate multicellular bacteria from such diseased/abnormal mammalian tissues *in vivo*.

Multicellular bacteria of the present invention may be used to produce *in vitro* industrial/commercial enzymes, *e.g.* polymer-degrading enzymes, and other proteins of industrial significance.

Multicellular bacteria of the present invention may be used to produce *in vitro* proteins as markers to diagnosis various mammalian diseases and pre-disease states.

The multicellular bacteria of the present invention may be used to produce *in vitro* protein and gene microarrays or biochips to aid in the diagnosis of multicellular bacterial infections associated with cancer, AIDS, and other human diseases and predisposition to disease (pre-disease states).

Brief Description of the Figures

Figure 1 shows tissue-like sheets and capillary-like networks of multicellular mammalian tumor-derived bacteria stained with the supravital dye new methylene blue N solution (Brecher formula) and examined by light microscopy. **a,** A tissue-like sheet contains lacunae. (liquid culture day 8) Scale bar, 500 μm.; **b,** Bacteria form a reticular, honeycomb-like pattern within a tissue-like sheet. (liquid culture day 7). Scale bar, 40 μm.; **c,** Bacteria form a capillary-like network that is attached to a tissue-like sheet (left upper corner). (liquid culture day 50). Scale bar, 250 μm.

Figure 2 shows a tissue-like sheet of multicellular mammalian tumor-derived bacteria heat-fixed, Gram-stained, and examined by light microscopy. Bacteria with a coccal morphology, single and in pairs, are stained purple with the Gram stain procedure[7] and, consequently, are Gram-positive by Gram-stain criteria and biochemical/16S

ribosomal DNA-based bacterial identification criteria. Many of the pleomorphic bacteria comprising the multicellular networks are stained red with the Gram stain procedure and, consequently, are "Gram-negative" by Gram-stain criteria although Gram-positive by biochemical/16S ribosomal DNA-based bacterial identification criteria. This finding suggests that their cell walls are deficient in peptidoglycan[7]. The largest of these pleomorphic bacteria have amorphous, undulating cell walls; the smallest appear as beads on a necklace and are a fraction of the size of a purple-stained coccus (~1 μm). In fresh preparations, these bead-like bacterial forms stain with the supravital dye new methylene blue N solution (Brecher formula) (liquid culture day 7). Scale bar, 20 μm.

Detailed Description of the Invention

Highly pleomorphic bacteria have been regularly isolated from mammalian tumors[3]. To determine if the pleomorphism of mammalian tumor-derived bacteria might be associated with eukaryote-to-prokaryote gene transfer[4], bacteria from canine lymphoma specimens were first isolated using a culture method that involves subjecting eukaryotic cells to an environmental pressure of alternating anaerobic and aerobic atmospheres[5] as follows:

1. Lymphoma specimens were collected sterilely from canines. (or – Tumor specimens are collected sterilely from mammals.)

2. Specimens were cut into several pieces with sterile scalpel/scissors in sterile conditions under laminar flow hood.

3. Specimens were placed into 5-8 ml of culture medium (bacteriological or eukaryotic) in sterile vented 60 mm Petri dishes in sterile conditions under laminar flow hood. The Petri dishes were covered.

4. Petri dishes containing specimens and media were placed in an anaerobic jar (Mitsubishi AnaeroPak jar), a gas generating pouch (Mitsubishi Pouch-Anaero) was added to the jar, and the jar was sealed to establish an anaerobic atmosphere.

5. The anaerobic jar was transferred to an incubator set at 37^0 C, and incubated for 24 hours.

6. The anaerobic jar was removed from the incubator, and the Petri dishes were removed from the jar.

7. The Petri dishes were opened and the specimens and media exposed to air for 10-15 minutes in sterile conditions under a laminar flow hood. The Petri dishes were covered.

8. Steps 4-7 were repeated.

9. A final anaerobic culturing cycle was performed as in steps 4 and 5.

10. The anaerobic jar was removed from incubator and the Petri dishes were removed from the jar.

11. All material (specimens and media) was transferred from the Petri dishes into bacteriological media bottles in sterile conditions under laminar flow hood.

12. The sterilely vented bacteriological media bottles were then transferred to an incubator set at $20\text{-}25^0$ or 37^0 C.

13. Bacteriological media bottles were observed daily for signs of turbidity.

14. When bottles became turbid, bottle contents were streaked onto solid media plates, and the plates were incubated for signs of microbial growth using standard conditions.

After isolation and culture on solid media, bacteria were identified taxonomically using biochemical methods and bacterial 16S ribosomal RNA (rRNA) gene sequence analysis[6]. All bacteria isolated were Gram-positive. Observing that several bacterial isolates appeared to self-organize *in vitro*, I selected one, identified as the Gram-positive

facultative anaerobe *Staphylococcus epidermidis* and designated as *MH*, for further morphogenetic study.

Within 72 hours of transfer from solid media into liquid culture, *MH* forms nonadherent, creamy-white, elastic material at the bottom of culture vessels. This property persists despite repeated liquid-solid-liquid media transfers. After several more days in culture, microscopic examination of fresh culture preparations stained with the supravital dye new methylene blue N solution (Brecher formula) reveals tissue-like sheets that contain lacunae (Fig. 1a). In some tissue-like sheets, reticular, honeycomb-like patterns are found in which the planar culture is tessellated with polygons with sides defined by cords of bacteria (Fig. 1b). In addition, capillary-like networks composed of strands of bacteria are found attached to tissue-like sheets (Fig. 1c) and floating free in the liquid medium. Bacterial strands in capillary-like networks recoil in turbulent medium, revealing their elastic properties.

Microscopic examination of Gram-stained tissue-like sheets at high magnification reveals a network of pleomorphic bacteria, many of which are "Gram-negative" by Gram-stain criteria (Fig. 2). The smallest bacteria appear as beads on a necklace and are a fraction of the size of a typical staphylococcus (~1 μm). Some triangular and quadrilateral areas circumscribed by this irregularly tessellated bacterial network are 3-4 μm^2. If present in the tumor microenvironment, bacterial networks of these dimensions might be difficult to detect using conventional histopathology techniques.

The multicellular self-organization *in vitro* of *MH* into tissue-like sheets and capillary-like networks shows some similarities to morphogenetic patterns observed in *in vitro* models of mammalian vascular network formation[8,9]. However, this multicellular

bacterial self-organization *in vitro* exhibits an autonomy and complexity of biological pattern formation not observed in these *in vitro* models of mammalian vascular network formation. Notably, while these models require extracellular matrix (ECM)-coated culture vessels to support endothelial cell self-organization, *MH* self-organizes *in vitro* in simple tryptic soy broth nonadherently and without the use of exogenous ECM. Multicellularity in such bacteria is defined as the cellular connection or interconnection of three or more bacteria into bacterial networks of varying densities that tessellate triangular, quadrilateral, and polygonal areas or shapes that contain no bacteria.

Studies using sequence-based comparative genomics indicate that some bacterial species appear to have acquired a significant number of genes from their eukaryotic hosts through horizontal gene transfer[4]. *MH* may express genes related to mammalian angiogenesis[10] that were acquired by way of eukaryote-to-prokaryote DNA transfer from canine eukaryotic tumor cells. Studies are underway to determine the type and degree of eukaryote-to-prokaryote DNA transfer that may account for the angiogenesis-like properties observed in *MH*.

As coevolved partners with animal eukaryotic cells, symbiotic bacteria have been shown to wield significant influence on animal development[11]. In symbiosis with mammalian eukaryotic tumor cells and mammalian capillary endothelial cells, *MH* and similar multicellular tumor-derived bacteria would wield significant influence on mammalian tumor development. As symbiotic capillary-like facultative anaerobes, they would stimulate tumor neovascularization, functioning as 'roadmaps' that guide mammalian endothelial cell migration into anaerobic regions of the tumor microenvironment; the bacteria *Bartonella henselae* and *Agrobacterium tumefaciens* have

been shown to trigger neovascularization in humans and plants, respectively[12,13]. One 'ecological' benefit of the interaction of multicellular tumor-derived bacteria with mammalian eukaryotic tumor cells and mammalian capillary endothelial cells would be the generation of an optimized bacterial habitat in which the bacteria would be assured a nutrient supply and host immune tolerance[11,12].

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I claim:

1. A culture of bacteria derived from mammalian tissues, which exhibit tissue-level multicellular self-organization when cultured *in vitro*.

2. The culture of bacteria of claim 1, wherein the bacteria exhibit sheet-like tissue-level multicellular self-organization cultured *in vitro*.

3. The culture of bacteria of claim 1, wherein the bacteria exhibit capillary-like tissue-level multicellular self-organization when cultured *in vitro*.

4. An autologous or allogeneic therapeutic vaccine to treat mammalian disease, produced from a culture of bacteria derived from mammalian tissues, wherein said bacteria exhibit multicellular self-organization when cultured *in vitro*.

5. The vaccine of claim 4, wherein the vaccine is a vaccine used to treat mammalian cancer.

6. The vaccine of claim 4, wherein the vaccine is a vaccine used to treat AIDS.

7. The vaccine of claim 4, wherein the vaccine is a vaccine used to treat cellular or tissue degradation associated with the aging process.

8. A method for screening drugs for efficacy against mammalian disease states comprising

a) exposing a culture of bacteria derived from diseased mammalian tissues, and that exhibit multicellular self-organization when cultured *in vitro*, to a candidate drug,

b) culturing said bacteria for a time sufficient to determine whether said bacteria are inhibited or killed by said candidate drug, inhibition or killing being an indicator of therapeutic efficacy of the drug.

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Application Data Sheet

Application Information

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Request for Non-Publication?::	No
Total Drawing Sheets::	4
Small Entity::	Yes
Petition included?::	No
Secrecy Order in Parent Appl.?::	No

Applicant Information

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[This application has no priority claims or assignee data]

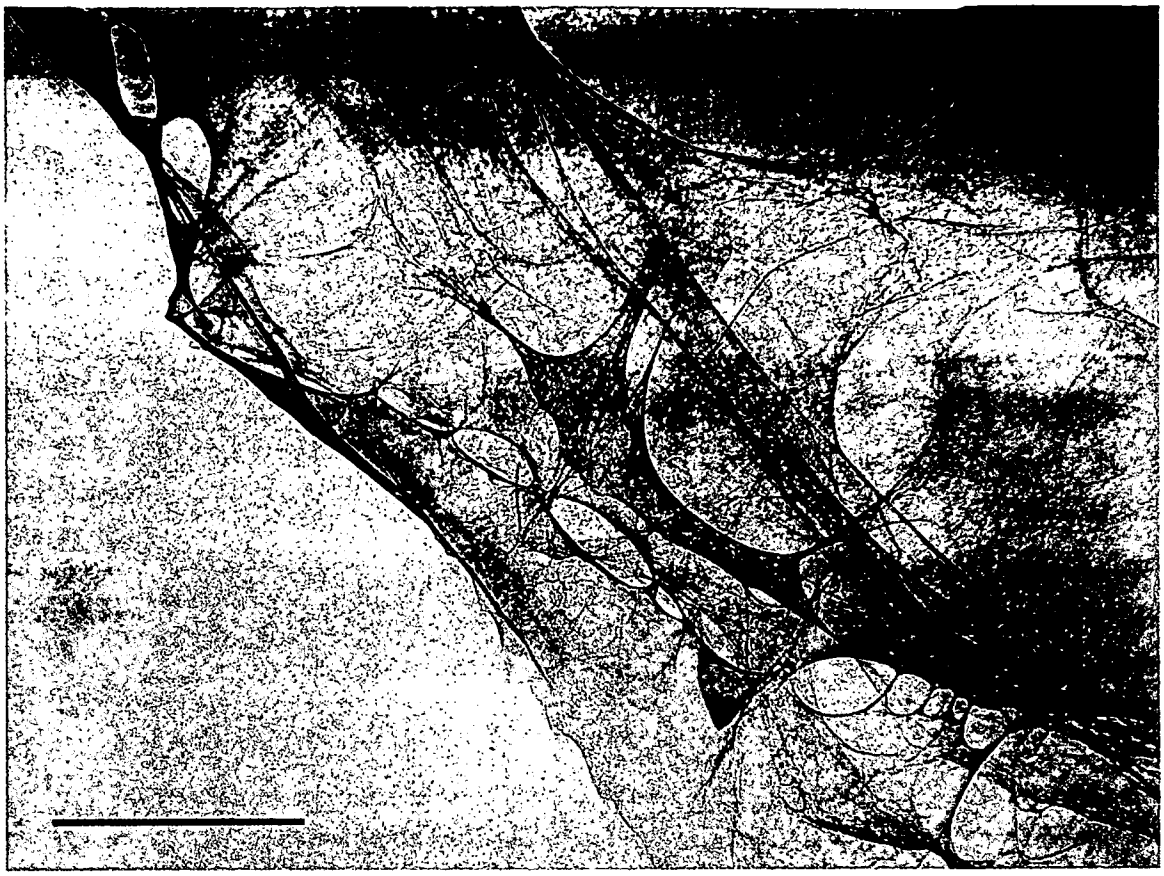

Figure 1a

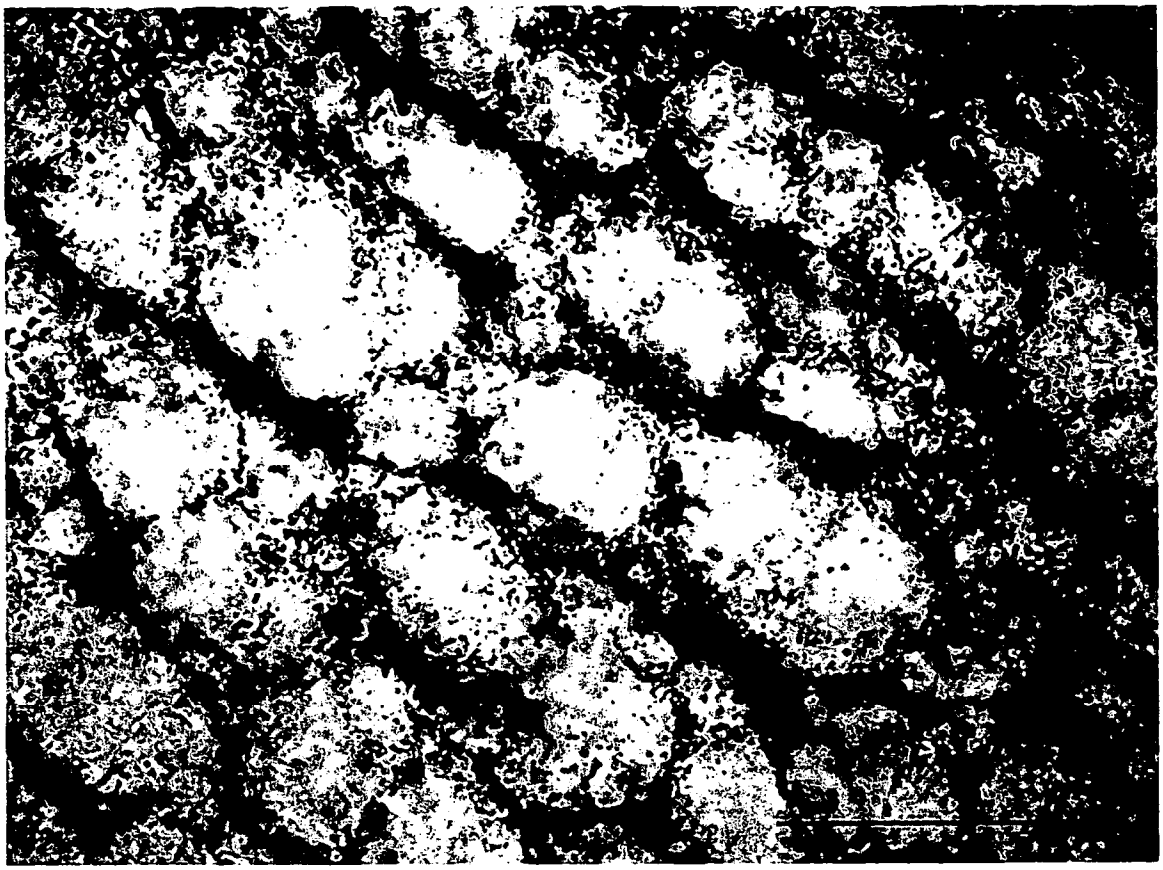

Figure 1b

Figure 1c

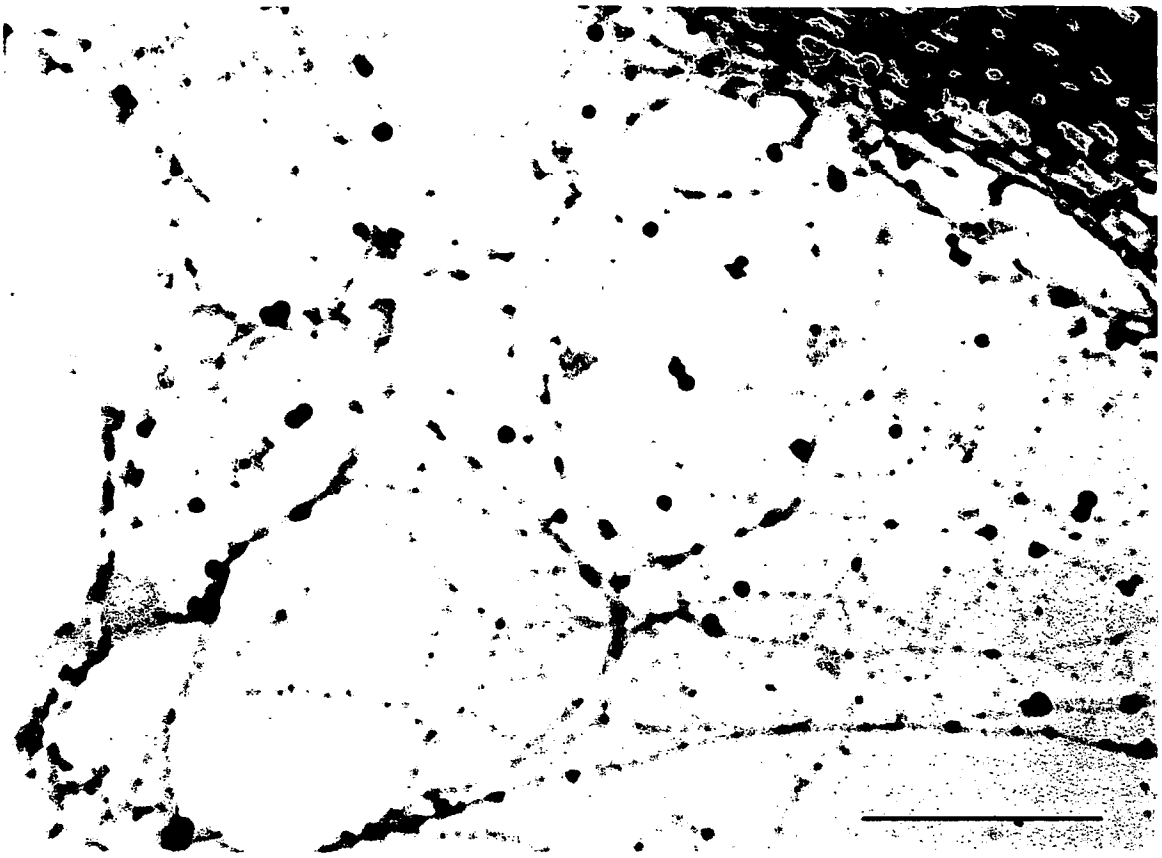

Figure 2